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(54) **Improved nucleic acid reagents and methods for their preparation**

(57) The invention is related to improved nucleic acid reagents comprising arrays of nucleic acid fragments and combinations of such fragments. The preparation of such fragments by recombinant DNA techniques and their use in hybridization methods is also described.

The improved nucleic acid reagents comprise two series, one labeled and one affixed to a solid carrier of at least two but preferably more arrays of alternating nucleic acid fragments, which are sufficiently homologous to sequences in the nucleic acid to be identified. Nucleic acid fragments belonging to different series must not be homologous to each other.

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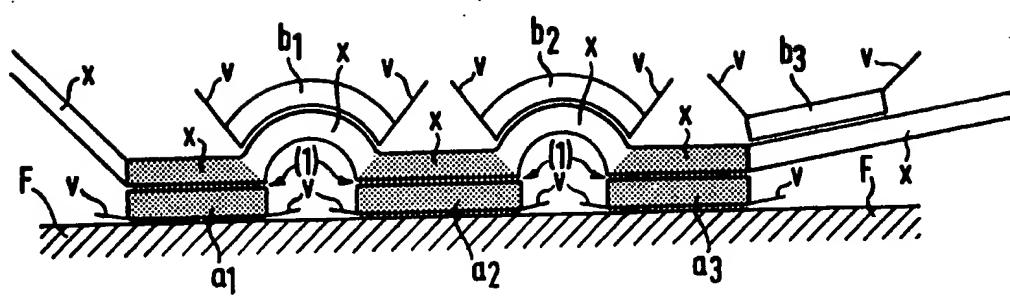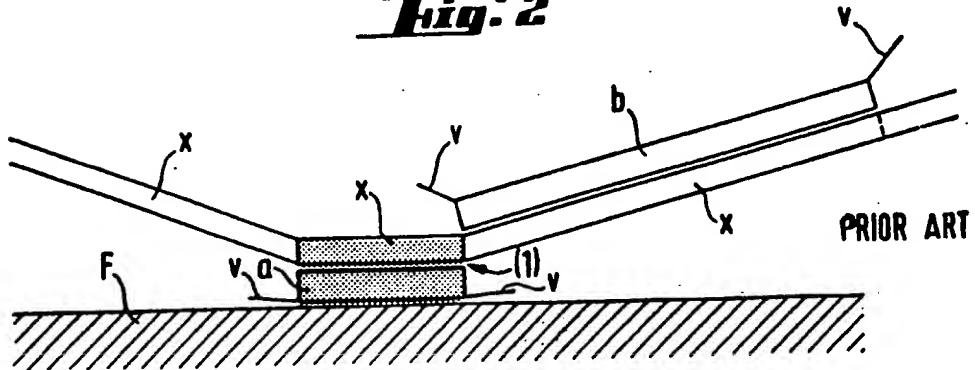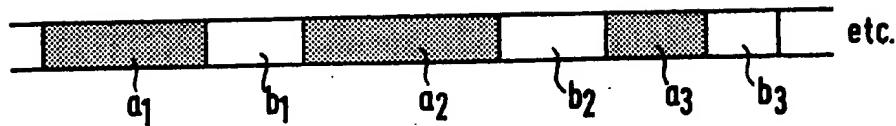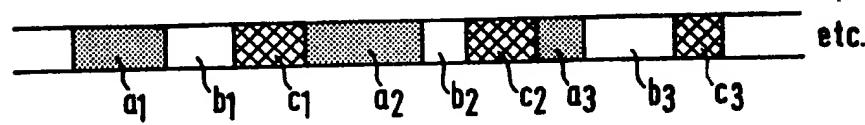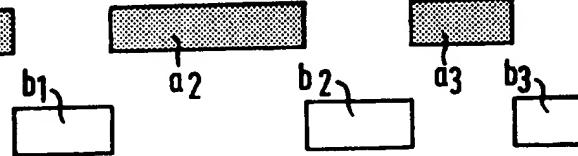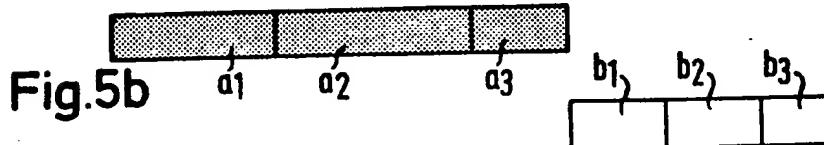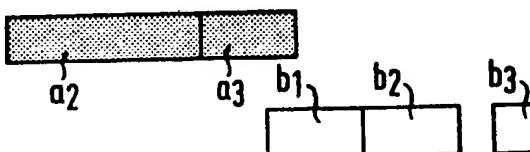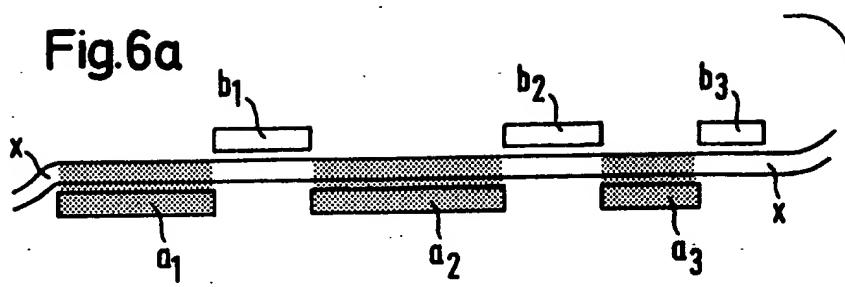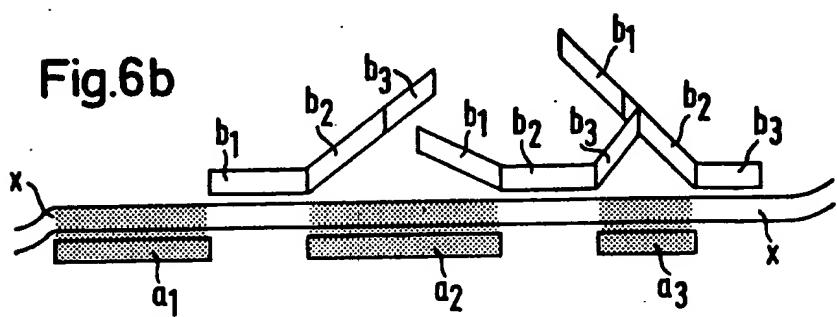
Fig. 1

Fig. 1 diagram showing cross-section with labels b_1, b_2, b_3, x, v, F, (1), a_1, a_2, a_3.

Fig. 2

Fig. 2 diagram showing cross-section with labels x, v, b, F, a, (1). PRIOR ART

Fig. 3

etc.

a_1 b_1 a_2 b_2 a_3 b_3

Fig. 4

etc.

a_1 b_1 c_1 a_2 b_2 c_2 a_3 b_3 c_3

Fig.5a a_1 b_1 a_2 b_2 a_3 b_3

Fig. 5

Fig.5b a_1 a_2 a_3 b_1 b_2 b_3

Fig.5c a_1 a_2 a_3 b_1 b_2 b_3

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Fig.6a

b₁   b₂   b₃

x
a₁   a₂   a₃

Fig.6b

b₃   b₁
b₂   b₃
b₁   b₂   b₃   b₂   b₃

x
a₁   a₂   a₃

x

Fig. 6

Fig.6c

b₁   b₂   b₁   b₂   b₃

x
a₁   a₂   a₃   x

Fig. 7

Aₓ   Aᵧ   A_z
v   v

a₁ₓ a₂ₓ a₃ₓ a₁ᵧ a₂ᵧ a₃ᵧ a₁_z a₂_z a₃_z

Fig.8a

Fig.8b

Fig.8c

Fig.8

Fig.9a

a_1 a_2 a_3

x

F

Fig.9b

b_1 b_2

x

F

Fig.9c

a_1 b_1 a_2 b_2 a_3

x

F

Fig.9

Fig. 10

Fig. 11

Fig.12

Eco RI

a₁

pKTH 1271

Eco RI

b₃

Bam HI

a₂

b₁   b₂

Pst I

Pst I   Cla I

Fig.13

mKTH 1277   mKTH 1278   mKTH 1279

b₁   b₂   b₃

X   X

X

a₁   a₂

pKTH 1273   pKTH 1274
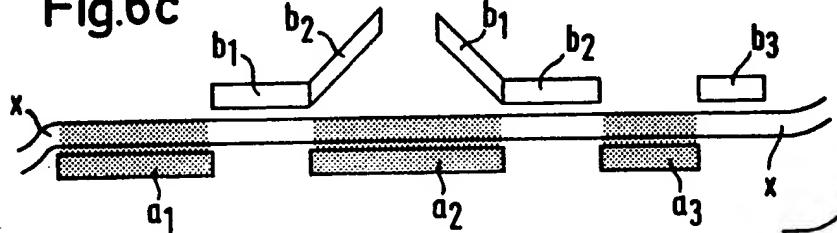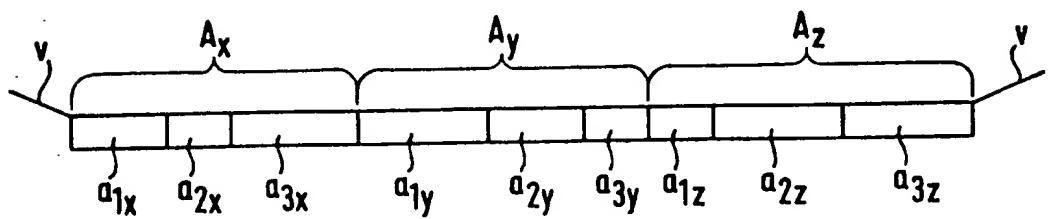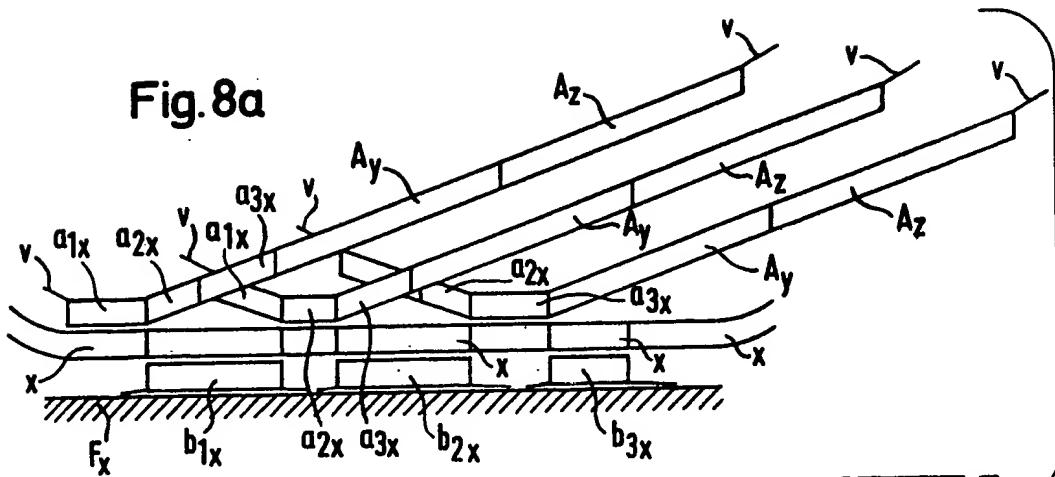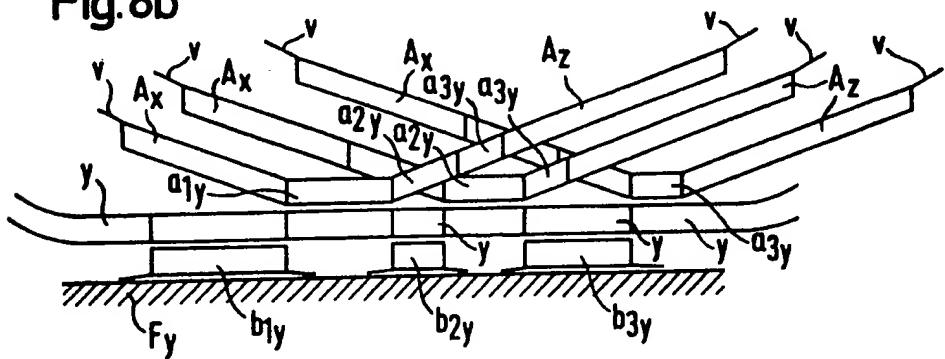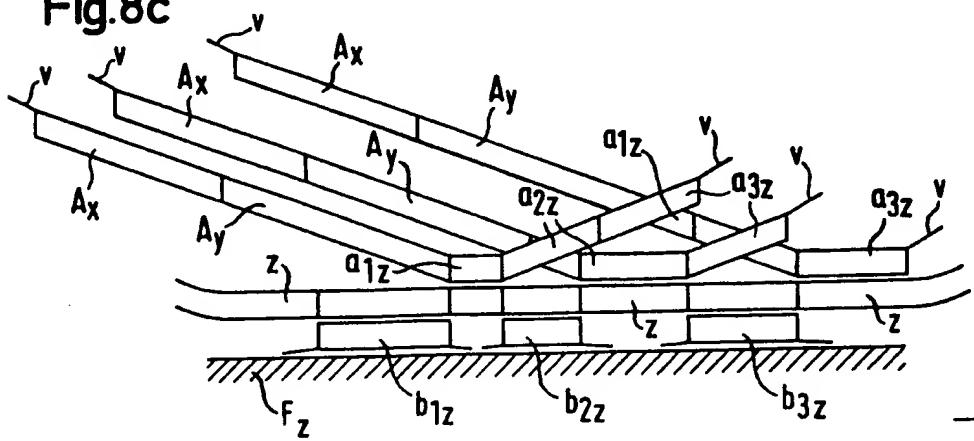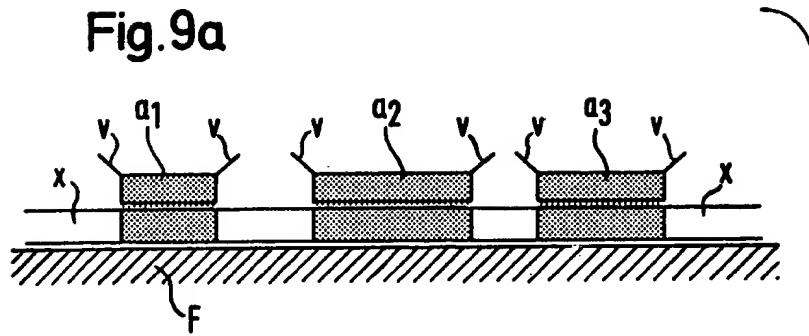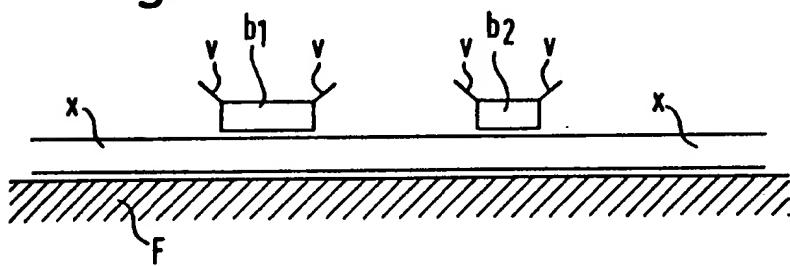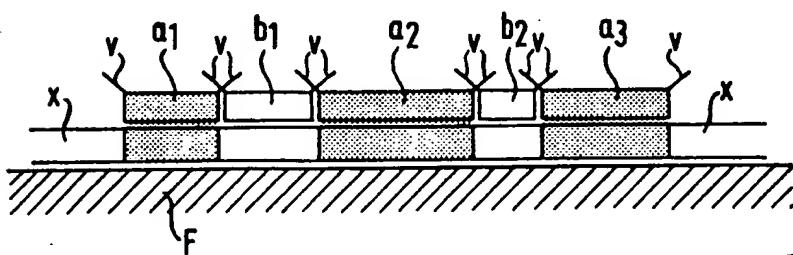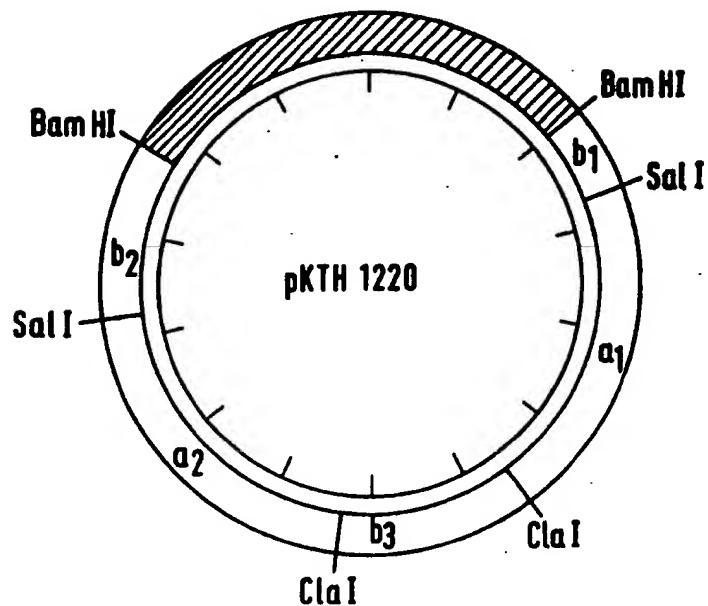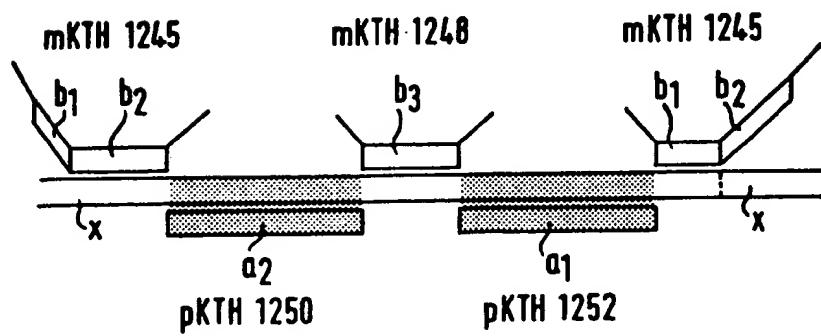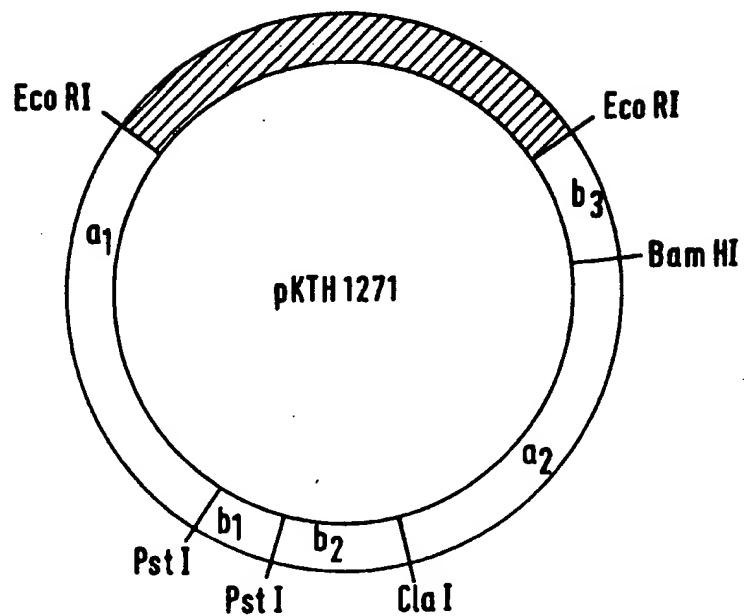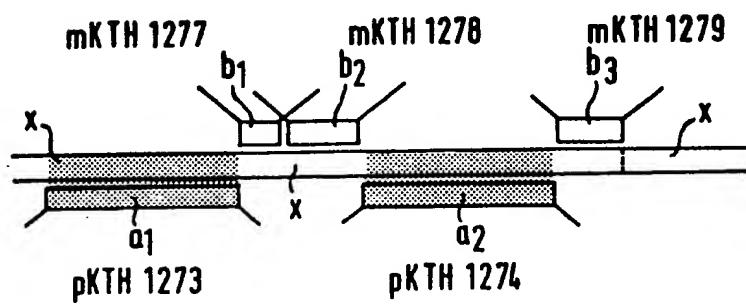
SPECIFICATION

Improved nucleic acid reagents and m thods for th ir preparation

5   The invention relates to improved nucleic acid reagents comprising an array of nucleic acid   5
fragments and to combinations of such improved reagents. The invention also relates to
methods for the preparation of nucleic acid reagents comprised of an array of clones, and
combinations of such nucleic acid reagents, by recombinant-DNA techniques, and to their use
for the identification of nucleic acids by hybridization methods.
10   Various hybridization methods have commonly been used for the identification and study of   10
nucleic acids. Some examples are the direct hybridization methods, in which the sample
containing the nucleic acid to be identified is either in a solution (Brautigam et al., J.Clin.Micro-
biol., 1980, *12*, 226–234 and the British Patent Publication No. 2,019,408) or affixed to a
solid carrier (US–Patent Nos 4,139,346, 4,302,204, 4,358,535, 4,395,486, the British
15   Patent Publications Nos. 2,034,323, 2,095,833, the European Patent Publications Nos   15
62,286, 62,237 and 61,740), and is detected by using one labeled nucleic acid reagent which
hybridizes with the nucleic acid to be identified.
Other known hybridization methods include the two-step sandwich hybridization method
presented by Dunn and Hassell in Cell, *12*, 23–36, 1977, and the one-step sandwich
20   hybridization methods presented in the European Patent Publication No. 79,139. For the   20
identification of the nucleic acids by the sandwich methods two separate nucleic acid reagents
are needed to detect the nucleic acids present in the sample solution. One of these reagents is
affixed to a solid carrier and the other is labeled.
Nucleic acid reagents, both those affixed to a solid carrier and those which are labeled, are
25   characterized in that their base sequence is complementary, or nearly complementary, to the   25
nucleic acid to be identified, i.e. homologous. The nucleic acid reagents used are either natural
nucleic acids as such or as fragments of them. The fragments are produced, for example, by
using restriction enzymes. Nucleic acid reagents have also been prepared synthetically or by
recombinant-DNA techniques. Natural plasmids (US–Patent No. 4,358,535), nucleic acids from
30   bacteriophages (US–Patent No. 4,543,535), ribosomal RNA and messenger RNA (US–Patent   30
No. 4,302,204), or nucleic acid from different viruses (Stålhandske et al., Curr. Top.Microbiol.
Virol. *104*, 1983) have been used as the nucleic acid reagents. The whole virus genome has
been used for identifying, for example, parts belonging to the different viruses in the messenger
RNA of a hybrid virus (Dunn and Hassell, Cell, *12*, 23–36, 1977). Nucleic acid reagents have
35   also been prepared by using recombinant-DNA techniques (US–Patents Nos 4,395,486 and   35
4,359,535, the European Patent Application No. 79,139 and the British Patent Publication No.
2,034,323 and the European Patent Application No. 62,286). Nucleic acid reagents produced
by recombinant-DNA techniques have been used either in such a way that the replicated defined
DNA fragment has been purified out from the DNA of the vector, or as recombinant-DNA
40   molecules linked to different vectors. The previously used nucleic acid reagents produced by   40
recombinant-DNA techniques are made up of one continuous identifying nucleic acid fragment
or of several separate clones.
We have developed new, more sensitive nucleic acid reagents, comprising at least two series
of alternating arrays of nucleic acid fragments prepared from either one or several segments
45   homologous to the nucleic acid to be identified.   45
Nucleic acid reagents which comprise such arrays of nucleic acid fragments are in sandwich
hybridization tests at least twice as sensitive as the previously used nucleic acid reagents. By
using the nucleic acid reagents according to the invention, or their combinations, it is possible to
identify smaller amounts of nucleic acids than previously, and they are especially well applicable
50   for sandwich hybridization methods.   50
The higher sensitivity of the nucleic acid reagents according to the invention in sandwich
hybridization methods is in part based on the fact that the use of several probes increases the
quantity of labeled hybrids on the solid carrier. There may be labeled vector-derived nucleic acid
along with every hybridizing probe (Figs. 1 and 2). In Figs. 1 and 2, v represents vector-derived
55   DNA, x the nucleic acid to be identified, b the labeled probe, a the identifying nucleic acid   55
reagent affixed to the solid carried, and F the filter. When several probes are used, the quantity
of labeled, vector-derived nucleic acid parts increases, and more label is bound to the hybrids
being formed. The hybrids are thus more easily detectable.
When the array of nucleic acid fragments according to the invention ar used in sandwich
60   hybridization m thods, at least two, or as shown in Fig. 1, three, identifying nucleic acid   60
fragments are affixed to the solid carrier. In this case the differ nt ar as of the nucleic acid
strand x to be detected may hybridize to the nucleic acid fragments affixed to the solid carrier,
f r xampl a_1, a_2, and a_3, at on r several points, depending on the degre of r action. Wh n
th reaction reaches its final stag , a situation according to Fig. 1 may b produced, in which
65   the sample strand forms a loop or loops to which the probe or probes, for example, b_1 and b_2 in   65

Fig. 1, hybridize. At this time th distanc of the vector-deriv d nucleic acid parts from the
hybridization joining point (1) d cr ases (Fig. 1), and th hybrid is more stable than th hybrid
formed by n reagent pair (prior art) shown in Fig. 2, this hybrid being of the same size as the
total area of the array of nucleic acid fragments. The vector-derived parts f a hybrid formed
5 from one reagent pair are easily broken by, for example, mechanical strain, such as shaking. In   5
such a case the label already bound to the hybrid escapes.
   Since the improved nucleic acid reagents according to the invention are more sensitive than
previously used nucleic acid reagents, they are suitable for demonstrating chromosomal
rearrangements and hereditary diseases.
10   Our invention relates to nucleic acid reagents comprising an array of nucleic acid fragments,   10
their combinations, their preparation, and their use for the detection of nucleic acids in
hybridization methods.
   The characteristics of the invention are shown in the distinguishing features of the claims, and
the invention is described in greater detail in the following description and in the acompanying
15 drawings, in which   15
      Figure 1 shows an array of sandwich hybrids,
      Figure 2 depicts a sandwich hybrid of the prior art,
      Figure 3 shows the sites of two alternating series of nucleic acid fragments in a nucleic acid
which has been selected for the preparation of an array of nucleic acid reagents according to the
20 invention,   20
      Figure 4 shows the corresponding sites of three alternating series of arrays of nucleic acid
fragments,
      Figure 5 shows an array of nucleic acid fragments according to Fig. 3 separate (a), joined
together (b) and both separate and joined together (c),
25   Figure 6 shows an array of sandwich hybrids,   25
      Figure 6a shows an array of sandwich hybrids which is formed when separate fragments are
used,
      Figure 6b shows an array of sandwich hybrid which is formed when joined b-fragments are
used.
30   Figure 6c shows an array of sandwich hybrids which is formed when bothseparate and joined   30
b-fragments are used.
      Figure 7 shows an array of nucleic acid reagents which identify different nucleic acids,
      Figure 8 shows an array of sandwich hybrids which are formed when the array of nucleic acid
reagents according to Fig. 7, identifying different nucleic acids, are used,
35   Figure 9 shows an array of hybrids formed by a direct hybridization method,   35
      Figure 10 shows the recombinant plasmid pKTH1220,
      Figure 11 shows an array of sandwich hybrids which is formed when an array of nucleic acid
  fragments prepared from the recombinant plasmid pKTH1220 are used,
      Figure 12 shows the recombinant plasmid pKTH1271,
40   Figure 13 shows an array of sandwich hybrids which is formed when arrays of nucleic acid   40
fragments prepared from the recombinant plasmid pKTH1271 are used.
   Our invention relates to nucleic acid reagents composed of an array of nucleic acid fragments.
These arrays of nucleic acid reagents comprise at least two, but preferably several, alternating
nucleic acid fragments, up to 20 fragments, which are derived from one or several nucleic acids
45 sufficiently homologous to the nucleic acid which is to be identified. Thereby there are obtained   45
at least two series of alternating arrays of nucleic acid fragments, which must not be
homologous to one another.
   The arrays of nucleic acid reagents can be prepared synthetically. In this case the fragments
from the two alternating series of arrays of nucleic acid fragments, must not be homologous to
50 each other. But they must be sufficiently homologous to alternating sites in the nucleic acids to   50
be identified. These fragments can easily be prepared by fully automatic machines after
characterization of the nucleic acid sequence of the nucleic acid to be identified.
   The nucleic acid reagents according to the invention are composed of separate, or joined, or
both separate and joined array of nucleic acid fragments.
55   The arrays of nucleic acid fragments may be joined to a vector, contain parts of vectors, or be   55
totally devoid of vector parts.
   The nucleic acid fragments used have a minimum length of 15 nucleotides. There is no actual
upper limit for length, but it is advantageous to use fragments having a l ngth of 20–5000
nucleotides. The nucleic acid fragments according to the inv ntion are deriv d either from th
60 genom to be identified or from one part of the genom , for example from a relatively large   60
clon representing a certain part f the genome. The arrays of nucleic acid fragments according
to the invention can thus be prepared from s veral independent genome areas which are not
directly adjacent. The arrays of nucleic acid fragments thus pr pared are combined and used for
the sam reagent. The arrays of nucleic acid fragments can also be isolated from a DNA which
65 is not id ntical to the nucleic acid to b identified but sufficiently homologous, so that a stable   65

hybrid is formed b tween the reagent and the nucleic acid to be identified. The preparation of
suitable arrays of nucleic acid fragments: is by no means limited to the isolation of suitable
nucleic acid fragments from the genom . There are available many equally us ful methods to
prepare such arrays of fragments. The man skilled in the art can prepare arrays of nucleic acid
5 fragments by synthetic or semisynthetic methods.   5
    The reagents are isolated in such a way that at least two series of alternating nucleic acid
fragments, a_1, a_2, a_3, etc., and b_1, b_2, b_3, etc., are obtained. The nucleic acid fragments
belonging to the series a_1, a_2, a_3, etc. are composed of fragments situated close to but not
adjacent to one another. The nucleic acid fragments belonging to the series b_1, b_2, b_3, etc. are
10 also composed of nucleic acid fragments situated close to but not adjacent to one another. The   10
nucleic acid fragments belonging to the series a_1, a_2, a_3, etc. and those belonging to the series
b_1, b_2, b_3, etc. must not be homologous to each other. It is preferable that the nucleic acids
belonging to the series a_1, a_2, a_3, etc. and those belonging to the series b_1, b_2, b_3, etc. are
isolated in such a way that every second fragment belongs to the a-series and every second to
15 the b-series, as shown in Fig. 3. In Fig. 3, a_1, a_2, a_3 and b_1, b_2, b_3 are arrays of nucleic acid   15
fragments sufficiently homologous to the nucleic acid to be identified. It is, of course, possible
that even a third nucleic acid fragment series, c_1, c_2, c_3, etc., is isolated from the same nucleic
acid, as shown in Fig. 4. It is preferable that the alternating two nucleic acid reagents follow one
another directly, but this is no absolute prerequisite for the invention.
20   The nucleic acid fragment series described above can be used either as separate fragments a_1,   20
a_2, a_3, etc., and b_1, b_2, b_c, etc. (Fig. 5a) or joined together into longer strands $a_1–a_2–a_3$, etc.,
and $b_1–b_2–b_3$, etc. (Fig. 5b). It is, of course, possible to prepare all kinds of intermediate forms
such as, for example, an a-series in which a_1 is a separate fragment and $a_2–a_3$ are joined
together, and in the b-series, for example, $b_1–b_2$ are joined together and b_3 is separate, etc., as
25 shown in Fig. 5c.   25
    Fig. 6 depicts various arrays of sandwich hybrids. Fig. 6a shows an array of sandwich hybrids
in which the arrays of nucleic acid fragments are separate. Fig. 6b shows an array of hybrids in
which the labeled array of nucleic acid fragments are joined together. Fig. 6c depicts a case in
which an array of sandwich hybrids is formed from both joined and separate labeled arrays of
30 nucleic acid fragments. In Fig. 6, x represents the nucleic acid to be identified; b_1, b_2, and b_3 30
represent the labeled probe, and a_1, a_2, and a_3 represent arrays of nucleic acid fragments affixed
to a solid carrier.
    Nucleic acid fragments which belong to the b-series can, for example, be labeled in such a
way that a labeled nucleic acid reagent is obtained, i.e. the probe B. The nucleic acid reagents
35 which belong to the a-series can be affixed to a solid carrier in such a way that a nucleic acid   35
reagent A bound to a solid carrier is obtained. It is, of course, alternatively possible to prepare a
labeled nucleic acid reagent A, and a corresponding nucleic acid reagent B bound to a solid
carrier.
    Such nucleic acid pairs A and B, or B and A, labeled and respectively affixed to a solid carrier
40 can be prepared for several different nucleic acids to be identified. They can be combined into   40
suitable nucleic acid reagent combinations, which are composed of different nucleic acid reagent
pairs A_1 and B_1, A_2 and B_2, A_3 and B_3, etc., or B_1 and A_1, B_2 and A_2, B_3 and A_3, etc. Reagents
containing arrays of nucleic acid fragments which identify different nucleic acids can also be
combined so that a probe $A_x–A_y–Z_z$ is obtained, which, for example, comprises an array of
45 nucleic acid fragments $(a_1–a_2–a_3)_x–(a_1–a_2–a_3)_y–(a_1–a_2–a_3)_z$, as shown in Fig. 7, in which a_{1x}, a_{2x} 45
and a_{3x} are arrays of nucleic acid fragments A_x which identify nucleic acid x; a_{1y}, a_{2y} and a_{3y} are
arrays of nucleic acid fragments A_y which identify nucleic acid y; a_{1z}, a_{2z} and a_{3z} are arrays of
nucleic acid fragments A_z which identify nucleic acid z, and v is a vector-derived nucleic acid
part. Joined arrays of nucleic acid fragments can, of course, also be used as separate fragments,
50 as suitable mixtures.   50
    The arrays of sandwich hybrids according to Fig. 8 are obtained by using the reagents shown
in Fig. 7. If simultaneous identification of several different nucleic acids is desired, it is, of
course, necessary to use separate filters, as shown in Fig. 8. Fig. 8a shows a solid carrier
identifying the nucleic acid x, Fig. 8b a solid carrier identifying the nucleic acid y, and Fig. 8c a
55 solid carrier identifying the nucleic acid z. In Figs. 8a, 8b and 8c, b_{1x} and b_{2x} are arrays of   55
nucleic acid fragments affixed to a solid carrier and identifying the nucleic acid x; b_{1y} and b_{2y} are
arrays of nucleic acid fragments affixed to a solid carrier and identifying the nucleic acid y; and
b_{1z} and b_{2z} are arrays of nucleic acid fragments affixed to a solid carri r and identifying the
nucleic acid z; and x, y and z are the nucleic acids to be identified. F_x, F_y and F_z are th
60 respective solid carri rs or filters, $A_x–A_y–A_z$ is a probe which identifies all the thr   nucleic acids   60
simultaneously, if separate solid carriers are used.
    The above-described nucl ic acid fragment series, reagents and reagent combinati ns can be
prepar d by recombinant-DNA techniques known *per se*. A number of nucleic acid fragments of
differ nt lengths are generated, by using restriction nzymes, from the nucleic acid to b
65 identified r from a part r pr senting it. If the restricti n map of the genome to b identified is   65

known, it is possible to select from the genom the suitable adjacent fragm nts, g nerated by
using restriction enzymes, and the fragments are isolated and amplifi d by using r combinant
DNA techniqu s.
When an unknown genome is involved, an intermediate stage can b us d in the preparation
5 of the reagents, in such a way that a relatively large restriction fragment is cloned, this fragment   5
is mapped, and the arrays of nucleic acid fragments series a_1, a_2, a_3, etc., and b_1, b_2, b_3 etc., are
produced on the basis of the information thus obtained.
It is, of course, possible to use combinations of the above methods and to use several large
separate cloned restriction fragments as starting material, and to prepare several separate series,
10 which are combined to form suitable combinations.   10
It is advantageous to prepare the nucleic acid fragment series a_1, a_2, a_3, etc., and b_1, b_2, b_3,
etc., according to the invention by using recombinant-DNA techniques in such a way that the
series a is cloned into one vector, for example into the plasmid pBR322, and whereas the series
b is cloned into another suitable vector, which does not have sequences in common with the
15 previous vector. The bacteriophase M13 is an example of such a second advantageous vector.   15
The fragments belonging to the series a can be joined to one another, and the joined series can
be cloned into one vector. For example, a_1–a_2, joined together, can be cloned as a continuous
insert into the same pBR322 vector. In a corresponding manner it is possible to prepare a
reagent series b_1–b_2. In the cloning it is preferred to use vectors to which very large inserts of
20 foreign DNA can be joined. For example, lambdaphage and cosmid vectors are suitable for this   20
purpose.
Thus, two reagent pairs comprising arrays of nucleic acid fragments are needed in the
sandwich hybridization method according to the invention, a reagent labeled with the label
substance to be identified, i.e. a probe, and a so-called filter reagent affixed to a solid carrier.
25 Most commonly, radioactive isotopes are used for labeling the probes. For example in the   25
British Patent Publication No. 2,034,323, the US–Patents Nos 4,358,535 and 4,302,204 the
following isotopes are used: ^{32}P, ^{125}I, ^{131}I and ^{3}H. In the European Patent Publication No.
79,139, the isotope ^{125}I is used. Nucleic acid probes have also been modified in different ways
and labeled with, e.g. fluorescent labels (French Patent Publication No. 2,518,755). Also
30 enzymatic or enzymatically measureable labels are used (the British Patent Publication No.   30
2,019,408, the European Patent Publication No. 63,879 and the French Patent Publication No.
2,519,005). The European Patent Publications Nos 70,685 and 70,687 describe a light-
emitting label and labeling method, and the French Patent Publication No. 2,518,755 describes
an immunologically measurable label. The lanthanide chelates described in US–Patent No.
35 4,374,120, especially europium, can be used as label substances. Also the biotin-avidin label   35
substance described by Leary et al. (PNAS *80*, 4045–4049, 1983) is suitable as a label. A few
examples of labels which can be used for the labeling of nucleic acid reagents according to the
invention are mentioned above, but it is evident that there will be developed new, improved
label substances which are also suitable for the labeling of arrays of nucleic acid fragments
40 according to the invention.   40
The carriers suitable for filter reagents include various nitrocellulose filters (US–Patent No.
4,358,535 and the British Patent Publication No. 2,095,833). The DDR–Patent Publication No.
148,955 describes a method of binding nucleic acids chemically to the carrier (paper).
US–Patents Nos 4,359,535 and 4,302,204 describe chemically modified papers which can be
45 used as solid carriers. Other alternatives include nylon membranes and modified nitrocellulose   45
filters. But it is evident that there will be developed new materials which will be even more
suitable for use as solid carriers according to the invention. It is, of course, possible to use also
other solid carriers, such as various chromatography matrices such as triazine- or epoxy-activated
cellulose, latex, etc. In principle, there are no other limitations to the selection of the solid carrier
50 than those to be described below. It has to be possible to affix nucleic acid in a single-stranded   50
form to the solid carrier so that these single-stranded nucleic acids can hybridize with the
complementary nucleic acid. The solid carrier must also be easy to remove from the
hybridization solution, or the hybridization solution must be easy to remove from the solid
carrier. Also, the probe must not adhere to the carrier material itself so that it cannot be washed
55 off.   55
The above-described combinations of the arrays of nucleic acid reagent pairs A and B, or B
and A, labeled and affixed to a solid carrier respectively, and from such nucleic acid pairs made
for the id ntification f different nucleic acids it is possible to assemble a combination A_x and B_x,
A_y and B_y, A_z and B_z.
60 Th s c mbinations can b used for th simultaneous identification of the nucl ic acids, x, y   60
and z by sandwich hybridization methods.
The sample is treated in such a way that the nucleic acids are released int the hybridizati n
solution, and they are rendered single-stranded. The hybridization is carried out in a hybridiza-
tion solution, to which both the nucleic acid reagents affixed to a solid carrier and the labeled
65 ones are added. When hybridizati n has taken place, the filters are lifted from the hybridization   65

solution, if filters have been used as solid carriers. If chromatography matric s, lat x, or the lik
have been used, th hybridization solution is removed. Th solid carriers ar rins d with a
suitabl washing solution. The arrays of sandwich hydrids formed (Figs. 8a, 8b, 8c) are detected
by methods known *per se*. The radi active label is measured, for example, by autoradiography,
5   by a scintillation counter or by a gamma-counter. For example, an enzymatic label is identified   5
after, for example, a color reaction, by photometry or on the basis of a precipitate. Lanthanide
chelates can be detected by a so-called "time resolved fluorescence" method. An immunological
label is detected by immunological methods suitable for the purpose.
     Several different mixtures can be used as the hybridization solution; the alternatives presented
10   in the European Patent Publication No. 79,139 and US–Patent 4,302,204 are mentioned as   10
examples. It is, of course, also possible to use other hybridization mixtures. The hybridization
takes place at a temperature of 0–80°C, but is advantageous to use, for example, a temperature
of 65°C. Sufficient hybridization may occur in a very short period, but it is advantageous to use
hybridization periods of, for example, 12–20 hours.
15   The two-step sandwich hybridization method is carried out in principle in the same manner,   15
but in this case the nucleic acid reagent affixed to a solid carrier is first added to the
hybridization solution. When the hybridization has taken place, the solid carrier is washed and a
second hybridization is carried out in which the labeled nucleic acid reagent is present.
     The above-described labeled nucleic acid reagents or reagent combination A_x, A_y, A_z, etc., and
20   B_x, B_y, B_z, etc., can, of course, be used in direct hybridization methods. In such a case the   20
nucleic acid sample in a solution must be divided for each nucleic acid x, y and z to be
identified or, if the sample is affixed to a solid carrier, a separate sample affixed to a carrier must
be prepared for each sample. The formed array of hybrids (Fig. 9) is detected by methods
known *per se*. In Figs. 9, F represents the solid carrier, i.e. the filter, x the nucleic acid to be
25   identified, and v the vector-derived parts. The labeled probes used are a_1, a_2 and a_3 (Fig. 9a), b_1 25
and b_2 (Fig. 9b), and a_1, b_1, a_2, b_2; a_3 (Fig. 9c).
     As already described above, various combinations of nucleic acid reagents can be made up
from the arrays of nucleic acid fragments according to the invention. It is possible by using
these combinations to identify several different nucleic acids simultaneously. Arrays of nucleic
30   acid fragments homologous to the different nucleic acids to be identified can be used as   30
separate fragments in the mixtures or joined together in such a manner that one probe
identifying several different nucleic acids is obtained. Nucleic acid reagents affixed to a solid
carrier must, of course, be kept separate in order for the identification to be successful.
     Hybridization using arrays of nucleic acid fragments can be used for identifying various
35   human, animal and plant pathogenic microorganisms. By the method it is possible to identify   35
microorganisms present in foodstuffs, such as clostridia, salmonellae, staphylococci, which
cause food poisonings. The method is suitable for the identification of contaminants present in
water, such as enterobacteria and enteroviruses.
     Since the sandwich hybridization test using arrays of nucleic acid fragments is a quantitative
40   method, it is applicable to, for example, the detection and measurement of gene amplification.   40
This characteristic is significant in, for example, the detection and treatment of cancer. The
formation of a stable array of hybrids requires that the homologous sequences of the probe
reagent and the filter reagent are located within a moderate, preferably less than 5 kilobase (kb),
distance from each other in the sample strand. If changes with respect to the distance between
45   these two areas do occur, the change is cleary observable by this method. Therefore the method   45
is also suitable for the detection of changed mRNA, chromosomal rearrangements, the
rearrangement of immunoglobulin genes for expression, and hereditary diseases. It is thus
possible to construct various reagent combinations from the arrays of nucleic acid fragments.
For example, for the identification of the causative agents of venereal diseases it is possible to
50   prepare kits which include a probe which contains arrays of nucleic acid fragments which   50
identify gonorrhea, syphilis, herpes and chlamydiae. The identification is in this case possible by
using separate filters for gonorrhea, syphilis, herpes and chlamydiae.
     The invention relates particular to arrays of nucleic acid fragments comprising the recombi-
nant plasmids pKTH1220 and pKTH1271. The recombinant plasmid pKTH1220 comprises, in
55   the plasmid vector pBR322, DNA of *Chlamydia trachomatis* L2 which is specific to the   55
Chlamydiae. This recombinant plasmid is cloned into the host *Escherichia coli* K12 HB101. The
recombinant plasmid 1271 comprises, in the plasmid vector pBR325, DNA from the cytomega-
lovirus AD169. This recombinant plasmid is cloned into host *Escherichia coli* K12 HB 101. The
hosts containing the recombinant plasmids pKTH1220 and pKTH1271 have b en deposited at
60   th culture collection Deutsche Sammlung von Mikroorganismen (DSM), Griesebachstrasse 8,   60
D–3400 Göttingen, West G rmany. The number of the deposit containing the recombinant
plasmid pKTH1220 is DSM2825 and the number of the deposit containing the r c mbinant
plasmid pKTH1271 is DMS2826. The deposits will be freely available onc th patent
application has b en made public.
65   The invention is described in great r d tail in the following examples. These exampl s must   65

not, however, be understood as limiting the protective scope of the invention. The structure of
the nucleic acid (DNA and RNA) is similar whether the question is of a nucleic acid derived from
a eucaryotic or a procaryotic cell. For this reas n the principles presented in the examples are
equally well applicable to the nucleic acids f animals (man included), plants and microbes or
5   viruses. Thus the reagents according to the invention can be used to detect the nucleic acids of   5
man, animals, plants, microbes and viruses. The arrays of nucleic acid fragments can be
prepared synthetically, too. The sequence of nucleic acids to be identified can be characterized
and homologous arrays of fragments prepared by automatic nucleic acid preparation machines.

10 *Example 1*   10
    (a)   *Arrays of nucleic acid reagents from Chlamydia trachomatis and their preparation*
    DNA fragments suitable for the diagnostics of the *Chlamydia trachomatis* group were prepared
from the DNA of *Chlamydia trachomatis* serotype L2. The DNA was isolated and fragmented by
known methods, and the resulting DNA fragments were cloned into the plasmid PBR322 and
15 transferred to the host organism *Escherichia coli* K12 HB101, by known methods. A gene bank   15
of the *Chlamydia trachomatis* L2 bacterium was obtained as a result of the cloning, i.e. a large
number of recombinant plasmids, each having a separate BamHI restriction fragment of DNA
derived from chlamydiae. For reagent production, recombinant plasmids containing maximally
large DNA inserts derived from chlamydial DNA were selected from the gene bank. One such
20 plasmid is the one designed pKTH1220, which has been deposited at the culture collection   20
Deutsche Sammlung von Microorganismen under the number (DSM 2825) and the suitability of
which for use as a reagent was demonstrated by a direct hybridization test. The test showed that
pKTH1220 identified all of the nucleic acids derived from different *Chlamydia trachomatis*
serotypes, but no other nucleic acids.
25 The applicable fragments, obtainable by using different restriction enzymes, were selected   25
from the pKTH1220-plasmid DNA, and some of these fragments were transferred by further
cloning into pAT153 plasmid (Maniatis et al., Molecular Cloning. A Laboratory Manual, Cold
String Harbor Laboratory, p.6, 1982) and some to M13 phage. Fig. 10 shows the recombinant
plasmid pKTH1220, having a molecular length of 14 kb. In Fig. 10, BamHI, SaII and ClaI
30 represent the restriction enzymes used, and a_1, a_2, b_1, b_2 and b_3 illustrate the size and mutual   30
locations of the fragments produced with the aid of these restriction enzymes. The fragments
belonging to the series b as labeled probes. Table 1 lists the sizes of the fragments and the
vectors used for further cloning, the names of the recombinant plasmids, and their use.

35 *Table 1.*   35

	Fragment	Size	Vector	Recombinant plasmid	Use
a_1	ClaI–SaII	3.0kb	pAT153	pKTH1252	Filter
a_2	SaII–ClaI	2.9kb	pAT153	pKTH1250	Filter
b_1	SaII–BamHI	0.7kb	M13mp8	mKTH1242	Labeled probe
b_2	BamHI–SaII	1.4kb	M13mp8	mKTH1239	Labeled probe
b_3	ClaI–ClaI	1.7kb	M13mp8	mKTH1248	Labeled probe
b1–b2	BamHI–BamHI	2.1kb	M13mp8	mKTH1245	Labeled probe

The fragments listed in Table 1 were isolated from an agarose gel by electroelution and were
cloned into the appropriate restriction enzyme identification sites of the vectors listed in Table 1,
50 by using known methods.   50
The fragment BamHI–BamHI 2.1kb was produced as follows: the fragments BamHI–SaII
1.4kb and SaII–BamHI 0.7kb of the plasmid pKTH1220 were separated by gel electrophoresis
in agarose gel, from which they were isolated. The purified fragments were joined to each other
with the aid of T4 ligase enzyme, and of the 2.1kb DNA fragments produced in the reaction,
55 those which had free ends which were identified by the BamHI enzyme were further joined to   55
the BamHI restriction site of the double-stranded form of the M13mp8 phage DNA. Thus there
was made a recombinant phage–DNA (mKTH1245) which contains *Chlamydia trachomatis* DNA
comprising two separate DNA fragments which are not locat d adjacently in the genome.
How ver, in the genom th y are locat d adjacent to the DNA reagents pKTH1250 and
60 pKTH1252 to be affixed to the filter (Fig. 11). Fig. 11 shows an array of sandwich hybrids   60
which is formed wh n th recombinant plasmids and recombinant phages listed in Table 1 ar
us d as arrays of nucl ic acid reagents.

    (b)   *Demonstration of the sensitivity of an array of nucleic acid reagents from Chlamydia*
65 *trachomatis by using the sandwich hybridization method*   65

The sensitivity of an array of nucleic acid reagents as compared with a single continuous reagent pair was studied by the sandwich hybridization m th d. The test was carried out using filters which all c ntained 10^{11} molecules of both pKTH1250 (a_2) and pKTH1252 (a_1) DNA rendered single-stranded. The sample to be studied was the plasmid pKTH1220, which for the
5   test was rendered single-stranded by boiling for 5 min in 0.17 M NaOH, whereafter it was   5
transferred to 0°C and neutralized with an equimolar amount of acetic acid. The following probes labeled with ^{125}J, listed in Table 1, were used in the tests: mKTH1242(b_1), mKTH1239(b_2), mKTH1248(b_3) and mKTH1245(b_1–b_2).
    The hybridization was performed at + 65°C for 17 hours in a hybridization solution having
10 the following composition: 4 × SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.2% SDS,   10
and 200 μg/ml herring sperm DNA. The filters were washed for 2 h at 50°C with a washing solution, having the following composition: 0.1 × SSC, 0.2% SDS, and were counted using a gamma-counter. The results are shown in Table 2 and are the means of five parallel tests.

15 **Table 2.**   15

Specimen	Hybridized radioactivity, with (b) as the probe					
molecules/test	b_1	b_2	b_3	b_1,b_2	$(b_1$–$b_2)$	$(b_1$–$b_2)$,b_3
0	37	37	33	49	39	52
10^6	48	44	48	93	68	140
10^7	226	236	232	396	416	686
108	1475	1415	1456	2912	2637	3580

b_1	380,000 cpm/test;	5 x 10^7 cpm/μgDNA
b_2	340,000 cpm/test;	4 x 107 cpm/μgDNA
b_3	350,000 cpm/test;	5 x 10^7 cpm/μgDNA
b_1–b_2	310,000 cpm/test;	7 x 10^7 cpm/μgDNA
b_1,b_2	700,000 cpm/test;	
$(b_1$–$b_2)$,b_3	700,000 cpm/test;	

    Statistically calculated, the 95% confidence limit of the tests performed without a sample
(= negative controls) was regarded as the lower limit for positivity. These values were 52–54 cpm when the probe was b_1, b_2 or b_3, 58 cpm when the probe was b_1, b_2, 56 cpm when the probe was b_1–b_2, and 65 cpm when the probe was b_1–b_2, b_3.

    (c) *Chlamydia diagnostics by using sandwich hybridization with arrays of nucleic acid fragments*
    Specimens taken from three men suffering from urethritis and three women suffering from cervicitis were selected for the test. *Chlamydia trachomatis* had been isolated from the male
55 urethral specimens and the female specimens taken from the cervix. In addition, a correspond-   55
ing number of similar patient specimens, from which chlamydia had not been isolated, were studied. The specimens to be examined were taken with cotton-tipped swabs which were immersed in a chlamydia sample-taking buffer containing 0.2 M saccharose, 20 mM phosphate buffer, 3% fetal calf serum, 10 μg/ml gentamicin, 100 μg/ml vancomycin, and 25 IU/ml
60 nystatin.   60
    Chlamydia was cultivated from the specimens. The original specimens wer also assayed by sandwich hybridization using an array of nucleic acid fragm nts. The specimens w re conc n-trat d by using 2-butanol to remove liquid from them in such a way that the final volume was about 80 μl, their concentration for th testing thus being about 3–7 fold. Thereafter 70 mA
65 EDTA, 0.7% SDS, 200 ug/ml prot inase K enzyme were add d to th sp cimen, and it was   65

treated for 15 min at 55°C and for 45 min at 37°C. Thereafter the specimen was boiled for 5
min in 0.175 M NaOH. The boiled specimen was transferred to 0°C and neutralized with an
equimolar amount f acetic acid and tested. The filters and hybridization conditions d scribed in
Example 1b were used in the test. The probe used was mKTH1245 (b_1–b_2), 300,000 cpm/400
5   μl hybridization reaction. The results ar shown in Table 3.   5

Table 3.

Specimen	Hybridized radioactivity	Result of chlamydia culture
Man 1.	151	+
Man 2.	164	+
Man 3.	154	+
Man 4.	61	−
Man 5.	76	−
Man 6.	55	−
Woman 1.	343	+
Woman 2.	509	+
Woman 3.	362	+
Woman 4.	57	−
Woman 5.	58	−
Woman 6.	81	−
Buffer, $\bar{X}_4$	30–55	
Chl. trachomatis L2 bacterium, 10^6	419	+

45   The limit for positivity in the tests was 104 cpm.   45
The result in Table 3 shows that sandwich hybridization using an array of nucleic acid
fragments is suitable for diagnosis venereal diseases. The samples which were negative in the
culture tests were negative also in the sandwich hybridization test.

50 *Example 2.*   50
(a)   *An array of nucleic acid reagents from Cytomegalovirus and their preparation*
DNA fragments suitable for the diagnostics of Cytomegalovirus were prepared from Cytomega-
lovirus (AD 169, ATCC VR–538)–(CMV). DNA was isolated and fragmented by known methods.
EcoRI fragment I of about 9 kb, defined in Spector et al., J. Virol. *42*, 558–582, 1982, was
55 isolated from agarose gel by electroelution after the EcoRI restriction fragments had been   55
separated on the basis of their size. The eluted DNA was extracted with phenol, whereafter it
was precipitated with ethanol. The DNA thus purified was joined by means of T4-ligase to the
pBR325 plasmid vector p ned by using the EcoRI enzyme, and the produced r c mbinant-
DNA w re transferr d to *E.coli* K12 HB101 host bacteria. From among ampicillin and tetracyclin
60 resistent but chloramphenicol sensitive clones ther was s lected on which contained a   60
cytomegalovirus-specific DNA insert of the correct size. The character of the cloned cytomegalo-.
virus DNA was ascertained by the South rn blot method. This test ensured that the d scribed 9
kb EcoRI–DNA fragm nt was derived from the DNA of Cytomegalovirus and, mor specifically,
was included in its HindIII–D fragment (Oram et al., J.Gen. Virol., *59*, 111–129, 1982). The
65 r combinant plasmid thus described was designated pKTH 1271, and it was deposited at the   65

culture collection Deutsche Sammlung von Microorganismen under number DSM 2826. The recombinant plasmid was grown and purified by known t chniques.

The further clonings were carried out by known techniques by using as vectors the pBR322 plasmid and the M13mp7 and M13mp8 phages. Figs. 12 shows the hybrid plasmid
5 pKTH1271 having a molecular length of about 9 kb. The array of nucleic acid fragments shown   5
in Fig. 12 were prepared by using the restriction enzymes EcoRI, BamHI, Clal and Pstl. Fig. 12 shows the fragments obtained by using the restriction enzymes as well as their relative size and location. Table 4 lists the sizes of the fragments in question and the vectors used for the further cloning, the names of the thus obtained recombinant plasmids, and their use either as filter
10 reagents or as labeled probes. Fig. 13 shows an array of sandwich hybrids which is formed   10
when the array of nucleic acid fragments listed in Table 4 are used.

Table 4.

	Restriction	fragment	Vector	Designation	use
a_1	EcoRI–Pstl	(3.3kb)	pBR322	pKTH1273	Filter
a_2	Clal–BamHI	(3.0kb)	pBR322	pKTF1274	Filter
b_1	Pstl–Pstl	(0.6kb)	M13mp7	mKTH1277	Labeled probe
b_2	Pstl–Clal	(1.0kb)	M13mp8	mKTH1278	Labeled probe
b_3	BamHO–EcoRl	(1.0kb)	M13mp8	mKTH1279	Labeled probe

(b) *Demonstration of the sensitivity of an array nucleic acid reagents from cytomegalovirus by*
25 *the sandwich hybridization method*   25
The sensitivity of an array of nucleic acid reagents as compared with one continuous reagent pair was assayed by the sandwich hybridization method. The specimen in the tests was CMV DNA, which was boild in 0.17 M NaOH for 5 min. and was thereafter neutralized as in example 1b. Filters which all contained 10^{11} molecules of both pKTH1273(a_1) DNA and pKTH1274(a_2)
30 DNA, rendered single-stranded, and the following probes labeled with ^{125}J listed in Table 4:   30
mKTH1277(b_1), mKTH1278(b_2) and mKTH1279(b_3) were used in the test. The probes each contained 10^8 cpm/μg DNA. The hybridization was carried out as described in Example 1b. The results are shown in Table 5.

35 Table 5.   35

Specimen	Hybridized radioactivity, with (b) as the probe				
molecules/test	b_1	b_2	b_3	b_1, b_2	b_1, b_2, b_3
0	35	33	38	45	53
10^6	38	44	46	95	125
4×10^6	85	135	142	205	292
1.6×10^7	203	254	265	415	645

b1	310.000 cpm/test
b2	320.000 cpm/test
b3	300.000 cpm/test
b1,b2	300.000 cpm of each/test
b1,b2,b3	300.000 cpm of each/test

65   In the test of value of the low r limit for positive was 51–55 cpm when the probe was b_1, b_2 65

or b_3, 59 cpm when the probe was b_1, b_2, and 63 cpm when the pr be was b_1, b_2, b_3.

The results in Table 5 show that sandwich hybridization in which an individual probe reagent in used (b_1, b_2 or b_3) detects in each case 4×10^6 CMV—DNA molecules. On the other hand, hybridization with a reagent of b_1, b_2 or b_1, b_2, b_3 detects as few as 10^6 molecules of
5 CMV–DNA. The r sults show that the array of nucleic acid reagents are four times as sensitive   5
as individual nucleic acid reagents.

(c) *CMV diagnostics by using sandwich hybridization with an array of nucleic acid reagents*
Clinical specimens were assayed by using sandwich hybridization with an array of reagents.
10 These samples included two urine specimens from children under 1 year. These children were   10
suspected of suffering from congenital cytomegalo disease. A lung biopsy specimen from a
patient with CMV pulmonary infection was also assayed by the present sandwich hybridization.
Both cytomegalovirus-infected and uninfected human fetal cells were also used as specimens in
the test.
15   A solution which contained 1% sarcosyl and 5 mA EDTA and 200 μg calf thymus DNA was   15
added to a 10 ml urine specimen, whereafter the DNA released from the virus, together with the
carrier, was precipitated using 10 ml isopropanol at room temperature. The DNA precipitate was
dissolved in 200 μl of TE buffer and was brought to a single-stranded form by boiling it for 5
min, whereafter the DNA solution was cooled to 0°C and added to the hybridization solution.
20   The lung biopsy specimen (a few mm³) was minced mechanically, with a knife, 200 μl of TE   20
buffer containing 1% SDS solution and 1 mg/ml of proteinase K-enzyme was added to it. A
digestion was carried out at +37°C for 1 h whereafter the specimen was drawn into an
injection syringe twice through a thin hypodermical needle. The specimen thus homogenized
was boiled, whereafter it was added to the test solution.
25   The cells infected with cytomegalovirus and the uninfected cells were broken up by an SDS,   25
proteinase K treatment, homogenized and boiled, as above.

The reagents in the hybridization test were pKTH1273(a_1) and pKTH1274(a_2) on filters and
mKTH1277(b_1), mKTH1278(b_2), mKTH1279(b_3) as probes, each 200.000 cpm/reaction. In
other respects the hybridization, the washing of the filters and the counting of the results were
30 carried out as described in Example 1b.   30
The results of the present hybridization are shown in Table 6.

Table 6.

Specimen	Hybridized radioactivity	Virus isolation
Infected cells (10^5)	3521	Not done
Urine 1(10 ml)	243	CMV
Urine 2(10 ml)	3215	CMV
Urine from a healthy person (10 ml)	52	Not done
Lung biopsy specimen	535	CMV
Control cells 10^5	68	Not done
No specimen	65	Not done

The results in Table 6 show that it is possible, by using an array of nucleic acid reagents, to
demonstrate CMV in different clinical specimens such as urine, lung biopsy specimens and cells.
50   The test is specific to cytomegalovirus; it does not identify human DNA, i.e. the test is not   50
interfered by the human DNA present in the sample. In fact the type of specimen does not
interfere with the specifity of test in any way.

CLAIMS
55   1. Nucleic acid reagents, characterized in that they comprise arrays of alternating nucleic   55
acid fragments.
2. Nucleic acid reagents according to claim 1, characterized in that they comprise two or
more series of at least two but preferably mor arrays of alt rnating nucl ic acid fragm nts
sufficiently homologous to the nucleic acid which is to be identified but not h mologous to ne
60 an ther.   60
3. Nucleic acid reagents according to claims 1 and 2, characteriz d in that th y c mprise
ither separat or joined arrays of alternating nucleic acid fragments.
4. Nucleic acid reagents according to claims 1, 2 or 3 characterized in that th y comprise
arrays of nucl ic acid fragments which either have or do not have v ctor-d rived parts.
65   5. Nucleic acid r agents accoording to claim 1, 2, 3 or 4 characteriz d in that they comprise   65

labeled arrays of nucleic acid fragments.

6. Nucleic acid reagents according to claims 1, 2, 3 or 4 characteriz d in that they comprise arrays of nucl ic acid fragm nts affixed to a solid carrier.

7. Nucleic acid reagents according to claims 1, 2, 3 or 4 characterized in that they comprise
5 the recombinant plasmid pKTH1220 or derivatives thereof and which recombinant plasmid   5
contains the DNA of *Chlamydia trachomatis* L2 bacterium and is cloned into the host *Escherichia coli* K12 HB101, and the deposit number of this host containing the recombinant plasmid pKTH1220 is DSM 2825.

8. Nucleic acid reagents according to claims 1, 2, 3, 4, 5 or 6 characterized in that they
10 comprise the recombinant plasmid pKTH1271 or derivatives thereof and which recombinant   10
plasmid contains the DNA of Cytomegalovirus AD169 and is cloned into the host *Escherichia coli* K12 HB101, and the deposit number of this host containing the recombinant plasmid pKTH1271 is DSM 2826.

9. The use of nucleic acid reagents according to claims 1, 2, 3, 4, 5, 6, 7 or 8 for the
15 identification of several different nucleic acids, characterized in that suitable combinations of   15
nucleic acid reagents are assembled from arrays of nucleic acid fragments sufficiently homolo-
gous to these different. nucleic acids.

10. The use of the nucleic acid reagents according to claims 1, 2, 3, 4, 5, 6, 7 or 8 in hybridization methods, characterized in that the arrays of hybrids formed in the hybridization
20 methods are demonstrated by methods known *per se*.   20

11. The use of nucleic acid reagents according to claims 1, 2, 3, 4, 5, 6, 7 or 8 in sandwich hybridization methods, characterized in that the arrays of sandwich hybrids formed in the sandwich hybrid methods are demonstrated by methods known *per se*.

12. A method for the preparation of nucleic acid reagents according to claims 1, 2, 3, 4, 5,
25 6, 7 or 8, characterized in that the arrays of nucleic acid fragments are prepared by   25
recombinant-DNA techniques, synthetically or semisynthetically.

13. A method according to claim 12, characterized in that the preparation of the arrays of nucleic acid fragments comprises:
(a)   the isolation of a selected nucleic acids of suitable length
30 (b)   the cloning of the selected nucleic acid into suitable vectors   30
(c)   the fragmentation of the nucleic acid by using a restriction enzymes
(d)   the combination of the suitable arrays of fragments into series by using suitable ligases
(e)   the cloning of the arrays of fragments into suitable vectors, preferably fragments
belonging to different series into different vectors
35 (f)   the labeling of the either separate or joined nucleic acid fragments belonging to one series 35
(g)   the fixation to a solid carrier of the either separate or joined nucleic acid fragments
belonging to the other series.

14. A method for the preparation of a nucleic acid reagent as claimed in claim 1, carried out substantially as hereinbefore described or exemplified.
40   15. A nucleic acid reagent as claimed in claim 1 and substantially as hereinbefore described. 40